Substituent Effects on Degradation Rates and Pathways of Cytosine Nucleosides

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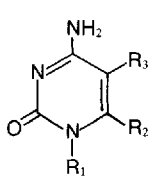
Abstract □ A previous report on the influence of a 6-methyl substituent on cytosine nucleoside degradation proposed that *N*-glycosyl hydrolysis predominated over the deamination pathway which was characteristic of the unsubstituted parent compounds. The UV absorption data which led to this hypothesis were not conclusive. Evidence for *N*-glycosyl hydrolysis was indirect and the product concentration was not quantitated. In the present study, specific HPLC methods were employed to assay four cytosine nucleosides and their corresponding bases, thus allowing comparison of the *N*-glycosyl hydrolysis rate to the overall rate of loss for each nucleoside. These data indicated that the 6-methyl nucleosides underwent partial or complete hydrolysis to yield their corresponding sugars and 6-methylcytosine, which then deaminated to 6-methyluracil. An increase in the reactivity and a change in the reaction products of the 6-methyl nucleosides were attributed to an alteration in conformation. In addition, the 6-methyl arabinosyl nucleoside reacted much faster than the 6-methyl ribosyl nucleoside, presumably due to 2'-OH participation. Degradation of 5-methyl deoxycytidine was also re-examined since its degradation was previously attributed solely to *N*-glycosyl hydrolysis. In the present study, simultaneous deamination and hydrolysis were measured, although *N*-glycosyl hydrolysis was found to predominate.

Cytosine derivatives as well as their nucleosides are known to undergo deamination to their corresponding uracils as illustrated in Scheme I. Consequently, the antileukemic agent cytarabine (1-β-D-arabinosylcytosine; araC) is inactivated by hydrolytic deamination to 1-β-D-arabinosyluracil (araU) during storage of solutions and by enzymatic deamination in vivo.[1,2]

Prior to the availability of HPLC, deamination rates of cytosine derivatives were studied by simultaneously measuring the decrease in the UV absorption maximum for the reactant and the increase in the UV absorption maximum for its deaminated uracil product.[2] Chemical kinetic studies using these techniques suggested that the deamination mechanism, which involved general base attack at the C-6 position, might be hindered by placing an alkyl substituent at the C-6



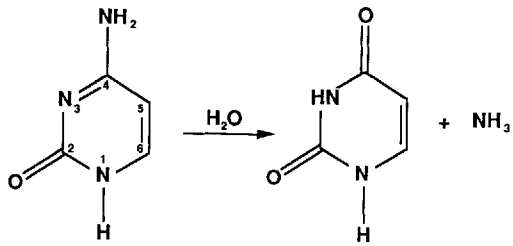
6-METHYLCYTOSINE: $R_1 = R_3 = H$; $R_2 = CH_3$

6-METHYLCYTIDINE: R_1 = ribosyl; $R_2 = CH_3$; $R_3 = H$

ARABINOSYLCYTOSINE: R_1 = arabinosyl; $R_2 = R_3 = H$

6-METHYLARABINOSYLCYTOSINE: R_1 = arabinosyl; $R_2 = CH_3$; $R_3 = H$

5-METHYLDEOXYCYTIDINE: R_1 = deoxyribosyl; $R_2 = H$; $R_3 = CH_3$



cytosine (C) uracil (U)

Scheme I

position.[2-4] To test that hypothesis, the degradation rates of 6-methylarabinosylcytosine (6Me-araC), 6-methylcytidine (6Me-Cyd), and 6-methylcytosine (6Me-C) were compared with those of the unsubstituted parent compounds by measuring the UV absorption at the maxima for the cytosines and their corresponding uracils as a function of time.[5,6] As predicted, 6Me-C was less susceptible to general base catalysis and 2–5 times more resistant to noncatalyzed deamination than cytosine itself.[5] In contrast, the absorption maxima decreased faster for 6Me-Cyd and 6Me-araC than for their parent compounds, but this decrease was not accompanied by the expected increase in UV absorption at the wavelength for the uracil chromophore. While these 6Me-nucleosides may have resisted deamination, this could not be assessed. It was suggested that the 6Me-nucleosides must have undergone competing reactions which were more rapid than deamination of the parent compounds.[5] Based on spectrophotometric, kinetic, and pK_a data, it was proposed that 6Me-Cyd and 6Me-araC had undergone partial or complete *N*-glycosyl hydrolysis to form 6Me-C and the corresponding sugars. However, a UV assay could not be developed for 6Me-C in the presence of the 6Me-nucleosides, since their UV characteristics are similar.

In the absence of a 6Me-C assay, *N*-glycosyl hydrolysis was not unequivocally established, especially since elimination of the sugar had not been previously reported for ribosyl or arabinosyl nucleosides of either cytosine or uracil.[2-12] It was therefore surmised that the 6Me substituent predisposed these nucleosides to *N*-glycosyl cleavage while stabilizing 6Me-C, which has no glycosyl moiety, to deamination.[5,6]

In order to test that hypothesis, the degradation kinetics have now been re-evaluated using HPLC methods which are capable of detecting the reactants, their bases (6Me-C and 5Me-C), and other reaction products. The specific aims of this research were: (1) to develop HPLC assays for several cytosine nucleosides which were previously studied by UV methods and for their corresponding glycolysis and deamination products (illustrated for 5Me-dCyd in Scheme II); (2) to determine

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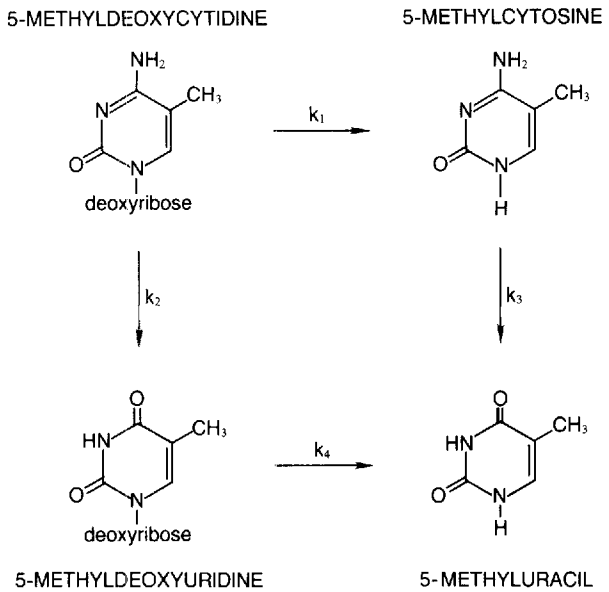


Scheme II

quantitatively and conclusively whether or not 6Me-Cyd and 6Me-araC undergo *N*-glycosyl hydrolysis to 6Me-C; (3) to determine the rate constants for *N*-glycosyl hydrolysis (k_1) and deamination (k_2) for each of the reactants; and (4) to use these results and those reported for other derivatives[5,11,13,14] to examine the influence of the glycosyl and the 5- and 6-methyl substituents on the competing rates for deamination and *N*-glycosyl hydrolysis.

Experimental Section

Materials and Methods—All reagents were analytical grade unless otherwise specified. 5-Methyl compounds were obtained from Sigma Chemical (St. Louis, MO), deoxyribose from Calbiochem (Los Angeles, CA); araC and araU from The Upjohn Company (Kalamazoo, MI), and cytosine and uracil from Nutritional Biochemicals Company (Cleveland, OH). The 6Me-U, 6Me-C, 6Me-araC, and 6Me-Cyd compounds were previously synthesized and reported.[5]

The temperatures, composition of the solutions, and pH values for the reactions are given in Table I. These experimental conditions were chosen to duplicate those in previous reports.[5,11] Temperatures were maintained within ±0.05 °C.

The initial reactant concentrations were 1.3 to 13 × 10^{-4} M. Reactions were initiated using each of the following reactants: 5Me-dCyd, 5-methyldeoxyuridine (5Me-dUrd), 5-methylcytosine (5Me-C), 6Me-Cyd, 6Me-C, araC, and 6Me-araC. Reactions were sampled as a function of time and diluted 10-fold with solutions which stabilized the samples throughout the time required for assay. The 5Me-dCyd reactions were diluted with 0.005 M acetic acid, 0.01 M sodium acetate, and 8% (v/v) methanol. Reactions initiated with 6Me-Cyd were diluted with 0.005 M NaH_2PO_4, 0.001 M Na_2HPO_4, and 5% (v/v) methanol. Reactions of araC and 6Me-araC employed 0.005 M acetic acid, 0.01 M sodium acetate, and 5% methanol. Samples from reactions initiated with the remaining compounds did not require stabilization. The pH values were measured at room temperature and extrapolated to reaction temperature.[15,16]

High-Performance Liquid Chromatography Assays—Reversed-phase HPLC assays were developed for 5Me-dCyd, 5Me-C, 5Me-dUrd, 5Me-U, 6Me-Cyd, 6Me-C, 6-methyluridine (6Me-Urd), 6Me-U, araC, C, araU, and 6Me-araC. A sample of 6Me-araU was not available. Table II summarizes the retention times, detection ranges, and capacity factors.

The mobile phases contained 0.005 M NaH_2PO_4 and 0.001 M $NaHPO_4$, with 8% (v/v) methanol for the 5-methyl derivatives and 5% (v/v) methanol for all other compounds. A fixed wavelength detector set at 254 nm with a 20-μL sample injection loop and a 25-cm analytical column were used (model 110A pump with model 153 Analytical Detector and Ultrasphere-ODS 5 μm, 4.6 × 250 mm; Beckman Instruments, Irvine, CA).

Figure 1 shows HPLC chromatograms representing relatively early and relatively late periods in reactions initiated with 6Me-Cyd, 6Me-araC, and 5Me-dCyd. The order of appearance of the products, the mass balance summing all assayed components as a function of time, and the data gained by initiating reactions using proposed intermediates were combined to establish the kinetic schemes discussed later. The detection of the *N*-glycosyl hydrolysis products (6Me-C or 5Me-C) allowed calculation of the competing rate constants for deamination (k_2) and *N*-glycosyl cleavage (k_1). Additional chromatograms and detailed discussions of the development of the HPLC assays are available.[17]

Hydrolysis of Deoxyribose—Seydel and Garrett[18] reported that 2-deoxyribose (DR) in 1.0 M HCl at 80 °C formed the degraded deoxyribose (DDR) chromophore with an absorption maximum at 261 nm within 5 h. The potential for assay interference by DR or DDR during the hydrolysis of 5Me-dCyd was examined by following the degradation of equimolar DR under identical conditions. Under the HPLC conditions employed, there was no peak observed for the DR standard. Conversely, DDR, formed by degrading a DR sample as described by Garrett and Seydel,[16] was determined by HPLC (Figure 1F).

Degradation of 5-Methyl Compounds—Apparent first-order rate constants for reactions initiated with 5Me-dCyd at pH 1.1 and 4.58 and with 5Me-dUrd at pH 1.1 were obtained from linear semilogarithmic plots of concentration versus time. At pH 4.58, where loss of 5Me-dUrd was slow, initial rate studies were employed. Four initial 5Me-dUrd concentrations in the range of 1.3 × 10^{-3} to 4.0 × 10^{-3} M were allowed to undergo ~10% loss. As a function of time, samples were collected, diluted, and assayed for 5Me-U. The first-order rate constant for 5Me-dUrd hydrolysis was obtained from the slope of a linear plot of initial rate versus initial 5Me-dUrd concentration.

Since the deamination rate of 5Me-C is extremely slow, initial rate studies were employed at both pH values. Data treatment was similar to that for 5Me-dUrd.

Degradation of 6-Methyl Compounds and 1-β-D-Arabinosylcytosine (araC)—The kinetics of loss of 6Me-Cyd, araC, and 6Me-araC

Table I—Rate Constants for Cytosine Nucleoside Transformations, Hydrolysis, and Deamination

Scheme	Reactant	pH	10^3k, min^{-1}					% *N*-Glycosyl Hydrolysis
			k_{obs}	k_1	k_2	k_3	k_4	
III	5Me-dCyd	1.14[a]	11.9[b]	11.8	0.131	0.0549; 0.0523[c]	1.43; 1.39[b]	99
		4.58[d]	1.35[b]	1.29	0.0533	0.0475; 0.0445[c]	0.0535; 0.0576[c]	96
IV	6Me-Cyd	3.63[e]	—	2.30	—	—	—	100
	Cyd	3.63	—	—	0.15[f]	—	—	0
	Cyd	1.1	—	—	0.10[f]	—	—	0
V	Ara-C	1.14	2.11[f]	—	2.11[g]	—	—	0
	Ara-C	3.63	3.76	—	3.76[g]	—	—	0
VI	6Me-AraC	1.14	23.1	12.6	—	—	—	55
	6Me-AraC	3.63	32.1	9.63	—	—	—	30

[a] 0.1 M HCl, 0.9 M NaCl; 5 Me-dCyd studies at 95 °C; all other reactions at 90 °C. [b] First-order plot. [c] Initial rate study, C_o ranges from 1.3 to 4.0 × 10^3 M. [d] Acetate buffer (0.06 M CH_3CO_2Na, 0.04 M CH_3CO_2H, 0.94 M NaCl). [e] Acetate buffer (0.80 M CH_3CO_2H, 0.08 M CH_3CO_2Na, 0.28 M NaCl). [f] Refs 4, 5, and 20. [g] Based on k_{obs} which reflects overall loss of reactant.

Table II—High-Performance Liquid Chromatographic Characteristics of Nucleosides and Bases[a]

Compound	Retention Time, min	Detection Range, 10^4 M	Capacity Factor
5Me-C	4.5	0.097–1.66	1.1
5Me-U	6.4	0.00293–0.0411	2.0
5Me-dCyd	9.1	0.0435–1.74	3.2
5Me-dUrd	14.2	0.00183–0.0252	5.5
Uracil	4.0	0.00112–0.00450	0.7
AraC	6.0	0.072–0.360	1.6
AraU	8.8	0.066–0.320	2.83
6Me-C[b]	5.7	0.026–0.242	1.3
6Me-U	7.6	0.00230–0.0151	2.0
6Me-AraC	6.5	0.0630–0.314	2.75
6Me-C[c]	5.7	0.364–3.64	1.3
6Me-Cyd	8.8	0.251–2.51	2.7

[a] Both mobile phases contained 0.005 M NaH_2PO_4 and 0.001 M Na_2HPO_4 with 8% (v/v) methanol used for 5-methyl compounds and 5% methanol for 6-methyl compounds and all unsubstituted compounds; flow rate was 1 mL/min. [b] Formation of 6Me-C from a 6Me-araC reaction solution at pH 3.63. [c] Formation of 6Me-C from a 6Me-Cyd reaction solution at pH 3.63.

in aqueous solutions were determined under pseudo first-order conditions at constant pH. All reactions were followed for a minimum of four half-lives. The hydrolysis of 6Me-U and araU at pH 1.1 was also studied for the period of time required for complete loss of 6Me-araC.

Results

5-Methyldeoxycytidine—The loss of 5Me-dCyd was accompanied by formation of 5Me-C, 5Me-dUrd, and 5Me-U, in that order, as illustrated in Figure 1E for 10 min and Figure 1F for 240 min. A typical concentration–time course, shown in Figure 2, is consistent with Scheme II in which k_1 and k_4 represent *N*-glycosyl cleavage and k_2 and k_3 indicate deamination. The scheme was further substantiated by initiating reactions with 5Me-C and with 5Me-dUrd. Degraded deoxyribose (DDR) was detected at pH 1.1, but not at pH 4.58. The percentage of $[5Me\text{-}dCyd]_0$ recovered by summing the time-dependent concentrations of 5Me-dCyd, 5Me-dUrd, 5Me-C, and 5Me-U was 95.0 ± 4%. Loss of 5Me-dCyd was described by apparent first-order kinetics: $d[5Me\text{-}dCyd]/dt = -k_{obs}t$, where $k_{obs} = k_1 + k_2$. The rate constant ratio was estimated from eq 1 during early periods when 5Me-C and 5Me-dUrd loss was insignificant:

$$k_1/k_2 = [5Me\text{-}C]_t/[5Me\text{-}dUrd]_t \quad (1)$$

The values calculated from the k_1/k_2 ratios and the first-order rate constants, $k_{obs} = k_1 + k_2$, were used as initial estimates for simultaneous nonlinear regression.

The 5MeC deamination rate constants (k_3) were calculated from the slopes of linear plots of v_o versus $[5Me\text{-}C]_o$ based on eq 2:

$$v_o \simeq k_3[5Me\text{-}C]_o \quad (2)$$

where v_o is the initial rate of 5Me-U formation.

At pH 1.1, the rate constant k_4 was obtained from a semilogarithmic plot of [5Me-dUrd] versus time. The HPLC assays also detected 5Me-U and DDR. However, at pH 4.58, only 5Me-dUrd and 5Me-U were detected. This was expected since DR cannot be detected by this assay and DDR formation requires HCl.[18] Since 5Me-U was the only product formed at pH 4.58, the initial rate was described by eq 3:

$$v_o \simeq k_4[5Me\text{-}dUrd]_o \quad (3)$$

where v_o is the initial rate of 5Me-U formation and k_4 was calculated from the slopes of v_o versus $[5Me\text{-}dUrd]_o$.

The concentration–time courses for 5Me-dCyd, 5Me-C, 5Me-dUrd, and 5Me-U in Scheme II may be expressed as follows:

$$[5Me - dCyd]_t = [5Me - dCyd]_o e^{-(k_1 + k_2)t} \quad (4)$$

$$[5Me - C]_t = \frac{k_1[5Me - dCyd]_0}{(k_1 + k_2 - k_3)}[e^{-k_3 t} - e^{-(k_1 + k_2)t}] \quad (5)$$

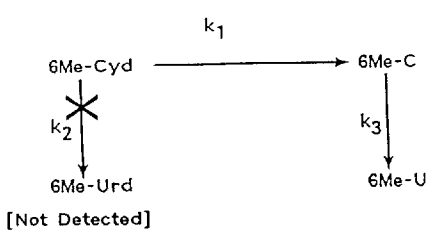
$$[5Me - dUrd]_t = \frac{k_2[5Me-dCyd]_0}{(k_1 + k_2 - k_4)}[e^{-k_4 t} - e^{-(k_1 + k_2)t}] \quad (6)$$

$$[5Me-U]_t = [5Me-dCyd]_o - \frac{k_1[5Me-dCyd]_o e^{-k_3 t}}{(k_1 + k_2 - k_3)} + \frac{k_1 k_3[5Me-dCyd]_o}{(k_1 + k_2)(k_1 + k_2 - k_3)} e^{-(k_1 + k_2)t} - \frac{k_2[5Me-dCyd]_o}{(k_1 + k_2 - k_4)} e^{-k_4 t} + \frac{k_2 k_4[5Me-dCyd]_o}{(k_1 + k_2)(k_1 + k_2 - k_4)} e^{-(k_1 + k_2)t} \quad (7)$$

Simultaneous nonlinear regression[19] was applied to the 5Me-dCyd, 5Me-dUrd, 5Me-C, and 5Me-U concentration–time course data (Figure 2) to evaluate the rate constants based on eqs 4 through 7. The values for k_3 and k_4 are similar to those determined directly using 5MeC and 5Me-dUrd (Table I).

6-Methylcytidine—First-order loss of 6Me-Cyd was accompanied by formation of the *N*-glycosyl hydrolysis product 6Me-C, as illustrated in Figure 1A. The sum of the time-dependent concentrations of 6Me-Cyd and 6Me-C provided a mass balance of 98 ± 3.6% during three half-lives of 6Me-Cyd loss. The deamination of 6Me-C to 6Me-U was detected after ~5 half-lives. The 6Me-Cyd deamination product (6Me-Urd) was not detected. Therefore, Scheme III describes the loss of 6Me-Cyd.

Arabinosylcytosine—The rate constant for loss of araC was obtained from a linear semilogarithmic plot of [araC] versus time. The assay detected araC, araU, and uracil (U). The total recovery of these three time-dependent concentrations was 85.4 ± 4.4%. The *N*-glycosyl hydrolysis product, cytosine, was not detected (Scheme IV). Deamination of araC has been reported to pass through a nonisolated intermediate of 20% maximum yield.[2] This is consistent with the ~15% unaccounted for by the HPLC mass balance.



Scheme III

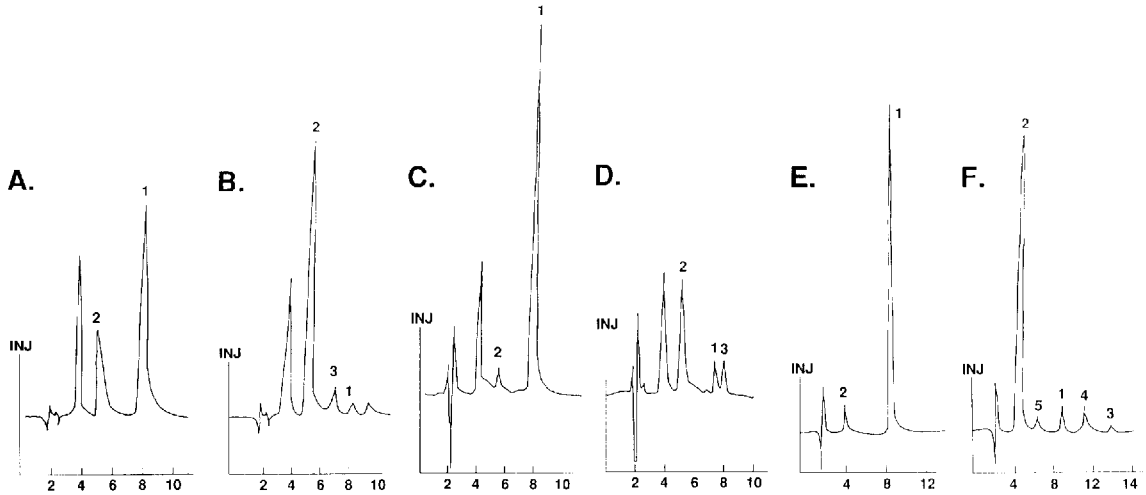


Figure 1—Examples of changes in HPLC chromatograms with time for the hydrolysis of 6Me-Cyd (pH 3.63, 90 °C) at (**A**) 3 h and (**B**) 25 h (#1 = 6Me-Cyd, #2 = 6Me-C, #3 = 6Me-U); for the hydrolysis of 6Me-araC (pH 3.63, 90 °C) at (**C**) 7 min and (**D**) 90 min (#1 = 6Me-araC, #2 = 6Me-C, #3 = an unidentified reaction product); and for the hydrolysis of 5Me-dCyd (pH 1.14, 95 °C) at (**E**) 10 min and (**F**) 240 min (#1 = 5Me-dCyd, #2 = 5Me-C, #3 = 5Me-dUrd, #4 = DDR, #5 = 5Me-U). Unlabeled peaks were also present in controls and were attributed to the buffers.

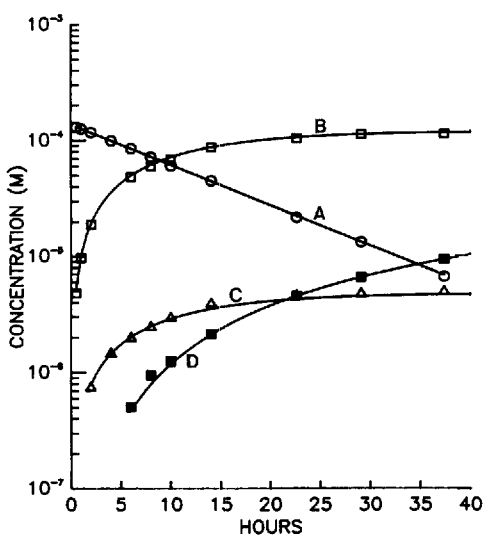
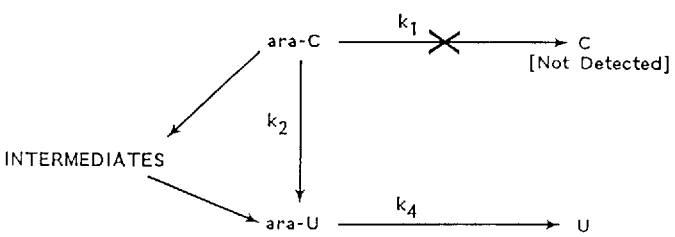


Figure 2—Semilogarithmic plot of the concentration–time courses of the components in Scheme II. Key: (A) 5Me-dCyd; (B) 5Me-C; (C) 5Me-dUrd; (D) 5Me-U. The nonlinear regression curves were obtained by simultaneously fitting the four sets of data to eqs 4–7.

6-Methylarabinosylcytosine—The formation of the *N*-glycosyl hydrolysis product 6Me-C (shown in Figure 1C) accounted for 55% of the first-order loss of 6Me-araC at pH 1.1 and 30% at pH 3.6. No other products were detected for two or more half-lives of 6Me-araC loss. The 6Me-U compound appeared late in the reaction in low yield (~1%) via deamination of the intermediate 6Me-C, which is known to form 6Me-U with a k_3 value of 2.52×10^{-5} min^{-1} at pH 3.63 and 90 °C.[5]

Following two reaction half-lives, an unidentified peak having a retention time of 9.0 min (Figure 1D) and a UV spectrum with an absorption maximum of 260 nm slowly formed as a function of time. This peak continued to increase after the loss of 6Me-araC was complete. Therefore, the unknown product must arise from an undetected intermediate. Scheme V shows the minimum number of reaction pathways required to describe these data. In spite of the unknown product, the quantitative



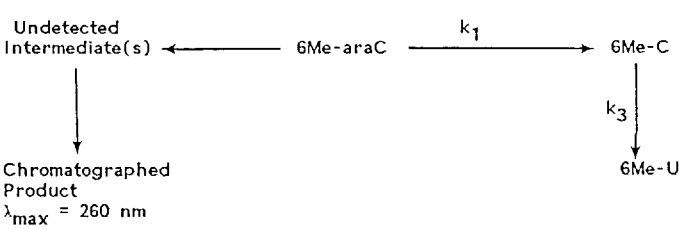
Scheme IV

determination of 6Me-C as a function of time allows the calculation of the *N*-glycosyl cleavage constant, k_1, from the observed first-order rate constant for total loss of 6Me-araC.

1-β-D-Arabinosylcytosine reportedly deaminated via a nonchromophoric intermediate which converted to araU when treated with mild alkali.[2] In the present study, no change in the chromatographed unknown product was observed when this treatment was applied to reaction mixtures. Since authentic 6Me-araU was unavailable, it could not be compared with the unknown peak. However, HPLC chromatograms of degraded nucleosides revealed that their deaminated products eluted immediately after the pyrimidine nucleosides themselves; therefore, the retention time (t_R = 9.0 min) of the unknown peak and its λ_{max} (260 nm) are physicochemical characteristics compatible with the identity of 6Me-araU.[17]

Discussion

When the initial studies using UV spectroscopy were published,[5] *N*-glycosyl cleavage had not been previously



Scheme V

reported for nucleosides with fully hydroxylated sugars such as ribose or arabinose.[2-9,20] Nucleosides containing deoxy sugars had been found to undergo *N*-glycosyl hydrolysis, but those reactions were slow even at high temperatures and low pH.[10-13] For a given base, the order of nucleoside reactivity was generally dideoxy > deoxy > hydroxylated, with the latter being sufficiently resistant to be regarded as stable in aqueous systems. The loss of the 6-methylcytosine nucleosides without formation of a uracil UV chromophore was therefore unexpected.[5]

Shapiro and Danzig,[11] using UV data, attributed the degradation of the deoxyribose analogue 5Me-dCyd entirely to *N*-glycosyl hydrolysis to form 5Me-C and deoxyribose. Recently, LC analyses showed that the fully hydroxylated ribosyl nucleosides cytidine, 5-bromocytidine, and 5-methylcytidine underwent ~50% deamination and ~50% *N*-glycosyl hydroylsis in HCl at 120 °C.[14]

It thus appeared that the kinetics and mechanisms of cytosine nucleoside degradation via deamination and sugar hydrolysis were influenced by the type of sugar and the 5- or 6-substituents. This conclusion is strengthened by the present study which re-examined several reactants (previously studied only by UV methods) by analyzing the *N*-glycosyl cleavage product, thus allowing the quantitative assessment of that rate constant.

Comparison of Rate Constants for 5-Methyl Compounds with Literature Values—The observed first-order rate constant for hydrolysis of 5Me-dUrd (k_4) agreed with the literature value at pH 4.58, but was three times larger at pH 1.1.[11] Interference by DDR in the literature assay is probably responsible for this discrepancy; DDR did not interfere with the present assay. At pH 1.1, DR degrades to DDR which has a λ_{max} at 261 nm.[18] In the literature assay, the DDR chromophore was reportedly destroyed by treating with 0.05 M NaOH and 0.5 M Na_2SO_4 for 16 h. Application of this treatment to DDR in the present study reduced the UV absorption only slightly. Loss of 5Me-dUrd (λ_{max} at 267 nm) was previously monitored by UV absorbances at 260 and 300 nm.[11] Interference by DDR would primarily elevate the 260 nm values, resulting in a negative error in the rate constant. Degraded deoxyribose (DDR) did not interfere with either 5Me-Urd or 5Me-U in the present HPLC assay.[17] Since DR did not form DDR at pH 4.58,[18] this k_4 value was in agreement with that in the literature.

The observed first-order rate constant for loss of 5Me-dCyd also agreed with the literature value at pH 4.58, but was slightly higher at pH 1.1. Since similar sample treatments were employed,[11,13] DDR would also explain this discrepancy. The formation of DDR during hydrolysis of 5Me-dCyd would not be as extensive as that during hydrolysis of 5Me-dUrd, since the 5Me-dUrd reaction time was 10 times longer at pH 1.1. Mass balance indicated that Scheme II quantitatively represented the reaction components. Thus, all of the rate constants in Scheme II were determined (Table I).

Substituent Effects on Degradation Rates and Pathways—Cytidine has been observed to quantitatively deaminate at 70–80 °C and pH < 7.[2,4] Similarly, araC deaminated to araU, although an intermediate was proposed in the pH region 0.5–4.5.[2] Using the present HPLC assay, the araC deamination products (araU and uracil) were detected, while the *N*-glycosyl hydrolysis product (cytosine) was not detected in the araC reactions. Thus, both Cyd and araC undergo complete deamination to their corresponding uracils.

Unlike Cyd, 6Me-Cyd degraded entirely by *N*-glycosyl hydrolysis to 6Me-C. The observed rate constant for this hydrolysis was 15 times greater than the deamination rate constant for Cyd at pH 3.63 (Table I). Thus, the 6-methyl substituent resulted in a less stable compound and completely altered the reaction pathway from deamination to *N*-glycosyl hydrolysis. This change in rate and pathway was also observed for 6Me-araC. The observed rate constant for loss of 6Me-araC is 8- to 10-fold greater than the deamination rate constant for araC (Table I). *N*-Glycosyl hydrolysis accounted for 30% of the initial 6Me-araC concentration at pH 3.63 and 55% at pH 1.1.

This change from deamination to *N*-glycosyl hydrolysis due to 6-methyl substitution has been suggested, but 6Me-C formation was not conclusive.[5] While nucleosides containing deoxy sugars underwent *N*-glycosyl hydrolysis in acid, those containing 2′-hydroxyl sugars exhibited greatly reduced rates.[21] Substitution of the base also markedly influenced the hydrolysis rate. Shapiro and Danzig[11] observed that 5-bromo-2′-deoxycytidine hydrolyzed 100 times faster than either 2′-deoxycytidine or 5-methyl-2′-deoxycytidine. This enhancement was attributed to electron withdrawal by bromine, making the base a better leaving group.

The 6-methyl substituent on Cyd and araC appears to activate *N*-glycosyl hydrolysis while inhibiting deamination. Inhibition of deamination by 6-methyl substitution has been previously discussed and verified by showing 6MeC to be 2–5 times more stable than cytosine.[5] This report shows that 6Me-Cyd and 6Me-araC are lost to *N*-glycosyl hydrolysis at greatly accelerated rates relative to the deamination of the parent nucleosides. Thus, even without assuming inhibition by the 6-methyl substituent, deamination could not compete with the accelerated loss via the alternate pathway. This difference in reactivity probably reflects steric factors. Proton and carbon-13 NMR studies indicated that pyrimidine base nucleosides exist preferentially in the anti conformation.[22] The 6-methyl group may force the nucleosides into a syn conformation. In the syn conformation, the 5′-hydroxyl group of 5Me-Cyd can hydrogen bond with the 2-carbonyl. Such an interaction would tend to attract electrons from the pyrimidine ring, making the base a better leaving group. The 6Me-araC compound reacts 10 times faster than 6Me-Cyd, presumably due to more favorable participation by its cis 2′-hydroxyl.[4]

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